

Review

Bioengineered 3D Models to Recapitulate Tissue Fibrosis

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Fibrosis, characterized by progressive tissue stiffening resulting in organ failure, is a growing health problem affecting millions of people worldwide. Currently, therapeutic options for tissue fibrosis are severely limited and organ transplantation is the only effective treatment for the end-stage fibrotic diseases with inherent limitations. Recent advancements in engineered 3D *in vitro* human disease mimic models, recapitulating the tissue pathophysiology, have provided unique state-of-the-art platforms for: (i) understanding the biological mechanisms involved in the disease pathogenesis; and (ii) high-throughput and reproducible drug screening. This review focuses on the recent multidisciplinary developments made towards advanced 3D biomimetic fibrotic tissue (liver, kidney, and lung) models that combine highly precision manufacturing techniques with high cellular functionality and biophysical (mechanical) properties.

Fibrosis

Fibrosis (see [Glossary](#)), characterized by an accumulation of excessive **extracellular matrix** (ECM) resulting in scar tissue and organ dysfunction, is a significant health problem affecting millions of people worldwide. With an increasing prevalence of obesity and diabetes, the incidence of fibrotic diseases and subsequently the number of fibrosis-related deaths will rise exponentially. Fibrosis is a common outcome of many chronic diseases in different tissues, including liver, kidneys, and lungs. Currently, there are no therapies available to reverse chronic fibrosis. Organ transplantation is the only option available for these patients, but organ shortage and chronic rejection limit the feasibility. The persistence of this unmet clinical need is due to the complexity of the disease process that involves numerous cell types and signaling pathways. Lack of reliable and reproducible, *in vitro* models recapitulating the pathophysiology of tissue fibrosis, has partially hampered the development of antifibrotic drugs. Therefore, in past years, tremendous advancements have been made towards the development of bioengineered fibrosis models, mimicking human disease pathophysiology, as reviewed here. Even though fibrosis plays an important role in many tissues, including heart [1] and skin [2], this review focuses on fibrotic liver, lung, and kidney tissue models.

Tissue fibrosis is a complex process, mainly characterized as a wound healing process triggered by chronic tissue injury ([Box 1](#)). Owing to this complexity, development of therapies has been challenging. Furthermore, therapies that have proved potentially effective in animal fibrosis models have failed in clinical trials, suggesting disparity between animal models and humans. This has led to the development of human disease mimicking *in vitro* models advancing from 2D monocultures/cocultures to self-assembled 3D spheroids and patient-derived **organoids**; from 3D-bioprinted and 3D-vascularized, bioreactor-based and microfluidically perfused **organ-on-a-chip** to tissue-based models including decellularized tissue scaffolds, precision-cut tissue slices (PCTS), and bioengineered artificial organs ([Figure 1](#), Key Figure and [Table 1](#)). These models recapitulating multiphenomenal tissue fibrosis, not only provide a versatile tool for disease modeling, but also aid in drug discovery by high-throughput drug screening in developed physiological *in vitro* disease models.

Highlights

Fibrosis, characterized by an excessive ECM deposition resulting in organ failure, represents a growing health problem worldwide. Where development of therapies is hampered due to disease complexity, recent advances in fibrotic tissue models generate promising platforms for understanding the disease and screening of new drugs.

Self-assembled 3D cultures are phenotypically stable models for studying fundamental cell-cell and cell-matrix interactions, amendable for high-throughput drug screening.

3D-bioprinted organotypic tissues resemble 3D spatial cellular organization and replicate key fibrogenic features.

Organ-on-a-chip and microfluidic systems combine 3D culture with controlled mechanical microenvironment, and recapitulate tissue fibrosis physiology.

Highly physiological *ex vivo* human tissue-based models closely mimic fibrotic tissues organization and composition.

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2D Models

2D models of fibrosis are still widely used as simple and reliable platforms for understanding the fundamental underlying disease mechanisms, and for high-throughput screening of pro- and antifibrotic compounds. 2D platforms provide several advantages such as ease of handling, multiplexing, high reproducibility, single-cell imaging and profiling (genomic, transcriptomic, and proteomic) under different culture conditions [3], based on which several fibrotic cellular markers have been identified.

Typical 2D cultures rely on the activation of primary rodent or human fibroblasts in monolayer on a tissue culture plastic (TCP). Primary fibroblasts, upon culturing on TCP or **transforming growth factor- β** (TGF- β) treatment, transdifferentiate into a highly proliferative, contractile, and ECM-producing **myofibroblasts** (MFs) resembling their respective *in vivo* counterpart [4]. In addition, single-cell analyses are widely used to isolate and characterize lineage-mapped MFs. Such techniques, based on single-cell RNA sequencing and transcriptome analysis, reveal the underlying molecular reprogramming mechanisms and unravel the fibroblast landscape in fibrotic diseases [5,6]. However, planar 2D culture platforms do not reproduce pathophysiological tissue fibrosis. Comparison of gene expression profiles has demonstrated that 2D-culture-activated **hepatic stellate cells** (HSCs) do not recapitulate the *in vivo* activated HSCs [3]. Furthermore, TCP is stiff (ranges in GPa) compared to *in vivo* conditions (ranges in kPa), suggesting that the mechanical environment in which these *in vitro* experiments are normally performed does not replicate tissue mechanics, which strongly influences cellular phenotype (Box 1). It has, for instance, been demonstrated that increasing ECM stiffness increases latent TGF- β 1 activation by MFs contraction [7]. Another major limitation of 2D models is their inability to mimic the 3D fibrotic microenvironment leading to physiologically irrelevant cellular behavior.

The physiological relevance of 2D platforms can be improved by: (i) tuning the mechanical environment; (ii) defining specific surface pattern on which cells can adhere and organize; and (iii) introducing 2D cocultures. Studies using mechanically tunable substrates have revealed that MF differentiation increases with increasing ECM stiffness. Using a tunable polydimethylsiloxane substrate, Balestrini and coworkers showed increased α -smooth muscle actin (SMA) expression, indicative of MF activation, on 100-kPa stiff substrates after 1 week of culture as compared to 5kPa soft substrates where they remain quiescent [8]. These ranges corresponded to the mechanical stiffness of fibrotic lung tissue (20–100 kPa) and normal lung **parenchyma** (1–5 kPa) [9].

A wide range of topographical surface patterns, defined as 2.5D cultures, have been introduced to reciprocate the physiological ECM and cell organization [10]. Furthermore, to increase complexity, a large number of 2D and 2.5D cocultures have been designed. Several studies have demonstrated that the direct **juxtacrine signaling** and indirect **paracrine signaling** interactions influencing cellular phenotype [11]. Pulmonary epithelial air–liquid interface culture, for instance, interfaces with lung microvascular endothelial cells and fibroblasts. These cocultures have enabled investigation of pathological paracrine signaling associated with different cell types [12,13].

3D Models

Even though 2D models have their merits, they are not able to replicate the physiological 3D tissue environment. Similar to *in vivo* conditions, 3D models enable cell–cell and cell–matrix interactions, cellular migration, chemotaxis, traction, and integrin adhesions, in all three planes, and replicate soluble growth factor gradients supporting cellular differentiation and maturation.

Self-Assembled 3D Systems

Perhaps the simplest 3D models are those that are attained by cellular self-assembly. Upon the combination of multiple cells in suspension or in a physically constraint environment like a

Glossary

3D biofabrication: use of additive manufacturing technology for the precise placement of biological materials and cells in 3D.

CRISPR/Cas9 gene editing: technique with which genomic DNA can be edited efficiently and highly selectively.

Extracellular matrix: network of extracellular macromolecules providing the structural and biochemical support for cells in a tissue.

Fibrosis: tissue stiffening as a result of excessive extracellular matrix deposition.

Hepatic stellate cells: when activated, the main cell type involved in liver fibrosis. Activated stellate cells are characterized by proliferation, contractility, and ECM deposition.

Hepatocytes: main functional cell type in the parenchymal tissue of the liver.

Interleukin-11: a downstream cytokine of TGF- β 1 signaling, associated with fibrotic diseases.

Juxtacrine signaling: cell signaling dependent on direct cell–cell contact.

Kidney glomeruli: network of small blood vessels (capillaries) that form the glomerular capillary bed and located in the nephron in the kidneys.

Liver sinusoids: type of fenestrated/porous blood vessel that form the capillary bed in the liver parenchyma.

Myofibroblasts: cells that are in between fibroblasts and a smooth muscle cells in phenotype. Myofibroblasts deposit vast amounts of ECM and are involved in tissue contraction.

Organ-on-a-chip technology: 3D cell culture platforms, based on microfluidic technology, that simulate the activities, mechanics and physiological responses of organs and organ systems.

Organoids: self-organized 3D tissue cultures that are derived from stem cells. Organoids contain multiple organ-specific cell types, which are spatially organized to replicate the organ organization, and are capable of replicating specific organ functions.

Paracrine signaling: cell signaling dependent on secreted factors.

Parenchyma: functional tissue of an organ as distinguished from the connective and supporting tissue.

Pericytes: cells that lie around and stabilize endothelial cells that line capillaries and venules.

microwell or hanging drop system, the cells aggregate to form 3D cellular structures. The lowest level of complexity is attained with cell aggregates composed of adult tissue cells or stem cell population, also referred to as spheroids [14]. Using a hanging-drop method, Bell and colleagues [15], have established and characterized phenotypically stable spheroids composed of primary human **hepatocytes** (PHH) and nonparenchymal cells (NPCs) [1NPC:2PHH]. These spheroids maintain proteomic levels for at least 5 weeks similar to those in the liver and retain interindividual variability. In another study, Leite and colleagues [16] developed spheroids of HepaRG hepatocytes and HSCs [1HepaRG:2HSCs], using low-attachment 96-well plates, which displayed the fibrotic features such as HSC activation upon single or repeated exposure (14d) to profibrotic compounds (allyl alcohol and methotrexate). Coll and coworkers [17] have developed highly reproducible induced pluripotent stem cell (iPSCs)-differentiated functional HSCs that remain quiescent when maintained as 3D spheroids with HepaRG, and become activated in response to hepatocyte toxicity resembling *in vivo* human HSCs, thereby generated a powerful tool for studying HSC development, disease modeling, and drug toxicity screening. Based on these HSC activation models, the biotech company Insphero has developed a standardized system of primary human liver spheroids, including Kupffer cells, amenable for antifibrotic drug screening in a reproducible high-throughput system (<https://insphero.com/applications/diabetes-and-nash-discovery/liver/fibrosis/>).

Organoids are more complex self-assembling structures of organ-specific cell types developed from stem cells or organ progenitor cells that follow sequential tissue developmental paradigms similar to *in vivo* [18,19]. As opposed to spheroids, organoids generally better replicate tissue shape and organization, and can contain multiple cell types that are distributed physiologically. Based on cell source, two different types of organoids can be distinguished; iPSC- and adult stem cell (ASC)-derived organoids [18,20].

iPSC organoids form complex organ structures following typically developmental processes and can be used to study disease development. Besides disease modeling, organoids provide a unique platform for high-throughput drug screening, personalized medicines, toxicology studies, and gene therapy. However, the development of iPSC-organoids is time consuming, due to limited cell expansion and maturation abilities. In contrast, ASC-derived organoids recapitulate adult tissue repair instead of development. Hence, only tissues with regenerative capacity such as the epithelium, are feasible for this approach. ASC-derived organoids form less-complex structures compared to iPSCs, but they can undergo several expansion passages within a short period of time, and are genetically stable, which makes them highly suitable for personalized gene-editing approach based on CRISPR-genomic differentiation.

Sachs and coworkers [21] have created long-term expanding human airway organoids containing basal, multiciliated, and secretory cells derived from lung cancer resections and metastasis biopsies from cystic fibrosis patients, showing a clear resemblance between *in vitro* cultured organoids and human disease pathophysiology. Wilkinson and colleagues [22] have generated self-assembled organoids with fetal lung fibroblasts, seeded on functionalized alginate hydrogel spheres using a rotational bioreactor. Upon TGF- β 1 incubation, these lung organoids show features of tissue scarring similar to idiopathic pulmonary fibrosis (IPF), and recapitulate the lung microenvironment when cultured with endothelial and epithelial cells. This shows that these organoids not only recapitulate tissue histology, but can also replicate a physiological tissue response. Comparably, Huang and coworkers [23] have generated 3D lung organoids via directed differentiation of developmental iPSCs into functional respiratory airway epithelial cells. Using **CRISPR/Cas9 gene editing**, HPS-associated interstitial pneumonia 1 (HPS1)^{-/-} 3D lung organoids were developed with HPS-associated interstitial pneumonia fibrotic disease

Perisinusoidal space or space of Disse: the narrow space between a hepatocyte and a liver sinusoid.

Transforming growth factor β 1: growth factor that is mainly involved in the regulation of inflammatory processes.

Tumor necrosis factor α : growth factor that promotes the systemic inflammatory response.

phenotype, clinically similar to IPF [24]. Furthermore, **interleukin-11** (IL-11) upregulation is observed in HPS1^{-/-} fibrotic organoids and IL-11 deletion prevents fibrosis development, demonstrating the efficiency of such a culture system in modeling fibrotic lung disease and identifying therapeutic targets [24].

Takasato and colleagues [25] have developed iPSC-derived organoids containing cells from multiple kidney lineages (comprising mesangial cells and **pericytes**), resulting in a promising mini-kidney model resembling the first trimester human kidney. Comparably, long-term expanding primary kidney tubular epithelial organoids, referred to as tubuloids, resembling proximal and distal nephron segments have been developed [26]. These can recapitulate kidney diseases, adopt a tubular conformation, and display active transport when cultured on a microfluidic organ-on-a-chip device.

Recently, by combining the protocol developed by Takasato with the NovoGen Bioprinter MMX technology, the biotech company Organovo has developed a robust platform for pharmaceutical drug testing. The inclusion of the bioprinter has allowed for the automated and scaled fabrication of human iPSC-derived kidney organoids without compromising their functionality (<https://organovo.com/wp-content/uploads/2019/07/2019-ISSCR-Organovo-poster-WHiggins.pdf>).

These 3D self-assembled systems therefore recapitulate numerous biological parameters including spatial cellular organization, cell–cell and cell–matrix interactions, and tissue-specific physiological functions. However, their future application is limited as these organoids resemble the primary substrate of disease, but lack mechanical cues, immune components, and perfused vasculature. Hence, even though these self-assembled systems can potentially model the cellular response to drugs, they are unlikely to replicate physiological disease progression and lack the capacity to model the infiltration of drugs via the blood.

3D Biofabrication

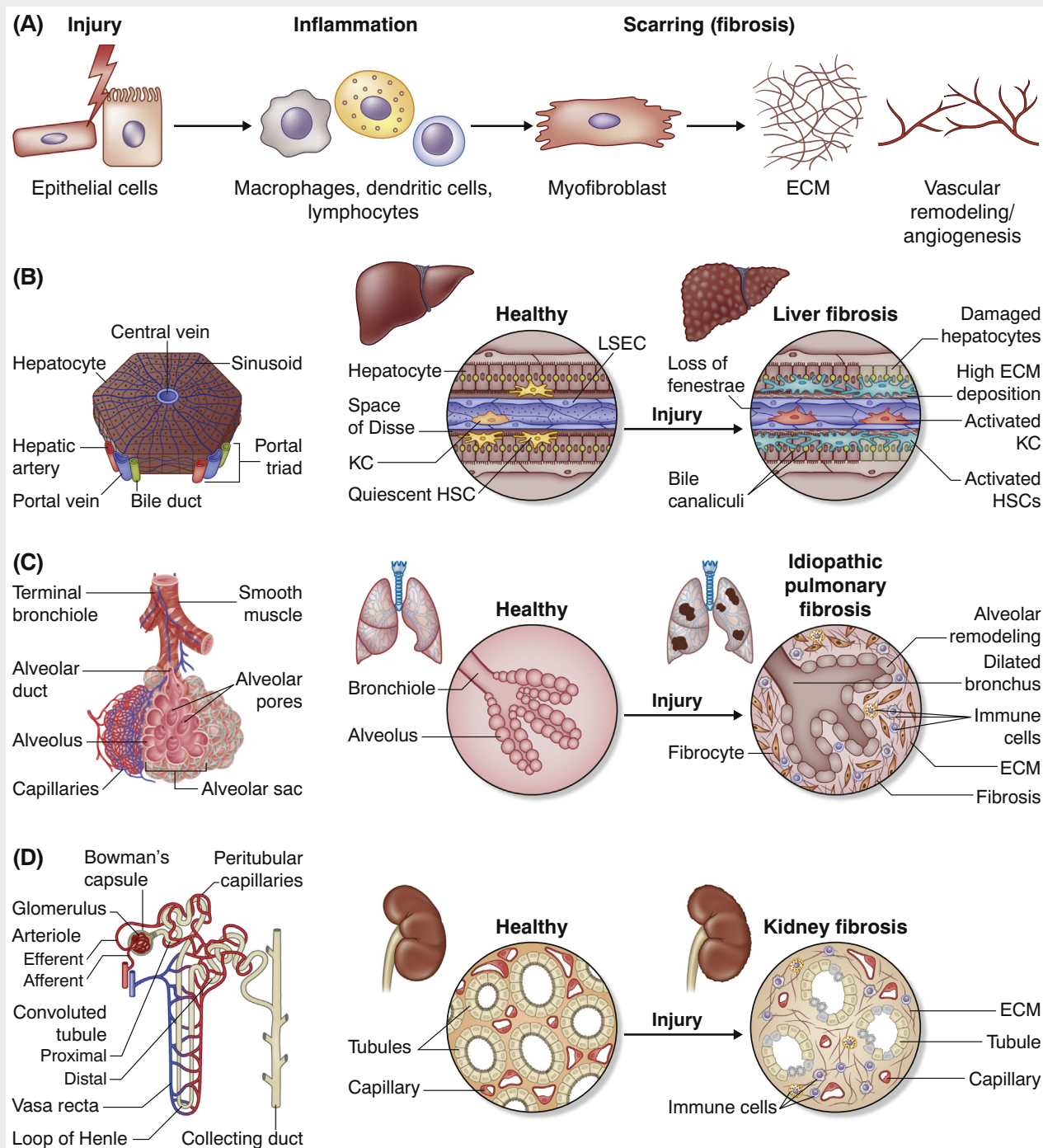
While organoids reproduce cellular organization using natural developmental processes, a precisely controlled process is often imperative, either to create a more physiological tissue

Box 1. Mechanisms of Pathological Fibrosis

Fibrosis is a wound-healing process initiated by chronic tissue injury followed by chronic inflammation and ECM deposition, ultimately leading to organ failure (Figure 1). Chronic injury to epithelial cells triggers the release of proinflammatory factors instigating the recruitment and activation of immune cells in the damaged tissue. Subsequently, these immune cells release profibrotic factors, such as cytokines, chemokines, and growth factors that cause the transdifferentiation of quiescent fibroblasts into activated MFs, identified by α -SMA expression. These cells secrete a vast amount of ECM components such as collagens resulting in tissue scarring and fibrosis [67].

MFs originate from different sources including tissue-resident mesenchymal cells, and epithelial and endothelial cells via epithelial-/endothelial–mesenchymal transition, pericytes and bone marrow (BM)-derived mesenchymal stromal cells (MSCs) [68,69]. In liver, the primary source of MFs are HSCs and portal fibroblasts [70]. Upon chronic injury, scar tissue deposition replaces the hepatocytes, causing loss of liver function [71]. In kidneys, the main sources are pericytes, interstitial fibroblasts, and glomerular mesangial cells. Upon chronic injury, glomerular and tubulointerstitial ECM accumulation leads to loss of organ architecture and function, leading to chronic kidney disease. In lungs, the major sources are interstitial fibroblasts, BM-derived fibrocytes, mesothelial cells, and epithelial alveolar cells. Chronic pulmonary injury results in IPF [72]. Furthermore, these tissues have significant amount of blood flow through specialized vessels including the **liver sinusoids**, **kidney glomeruli**, and pulmonary blood vessels. Altered flow through these vessels could be the cause and result of tissue remodeling and fibrosis leading to pathological angiogenesis [73].

Although most research has focused on MFs to understand disease mechanisms and design drug targeting approaches, other components such as immune (especially macrophages) and endothelial cells, soluble molecules (chemokines, cytokines, and growth factors, notably TGF- β) [74], hypoxia, matrix stiffness, mechanical strain/shear stress, and vascular and interstitial flow, have also been recognized to play an important role [75]. MFs cause ECM reorganization and contraction affecting cellular behavior in response to biochemical and mechanical stimuli [7,76]. Importantly, mechanical stimuli driven by physical microenvironment or cellular mechanosensing by different mechanisms involving calcium, mechanosensitive-sensitive ion channels, matrix mechanosensors (e.g., integrins), transcription mechanosensitive factors (e.g., YAP/TAZ), and myocardin-related transcription factor), are an important mechanism in organ fibrosis [77]. Fibrosis evolves from a concerted interaction between different cell types with tissue microenvironmental signals, involving autocrine, paracrine, juxtacrine, endocrine, angiocrine, mechanocrine, and neuroendocrine signaling.

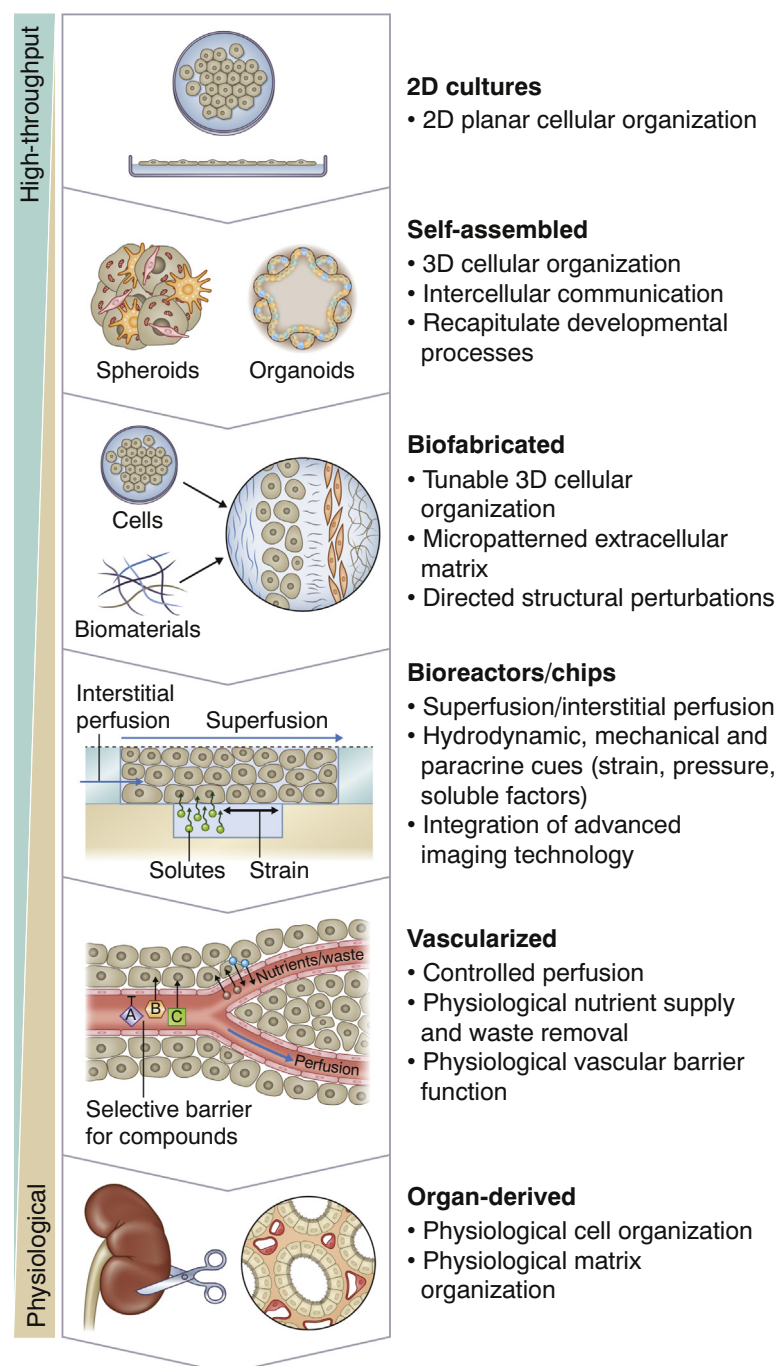


Trends in Biotechnology

Figure 1. Schematic Illustration Depicting Tissue Microstructures and Fibrosis Pathogenesis. (A) Schematic diagram presenting general pathogenesis of tissue fibrosis. Injury to epithelial cells instigate inflammation, that is, infiltration of immune cells. The inflammatory cells secrete cytokines and growth factors which activate local fibroblasts and infiltrated mesenchymal cells into myofibroblasts. Eventually, myofibroblasts secrete extracellular matrix (ECM) remodeling leading to scarring of the tissue and vascular remodeling/angiogenesis. (B) Schematic representation of liver lobule with changes in liver architecture during fibrosis. (C) Schematic diagram of lung alveoli and changes in the lung architecture during fibrosis. (D) Schematic representation of kidney tubule with changes in the kidney architecture during fibrosis. Abbreviations: HSC, hepatic stellate cell; KC, Kupffer cell; LSEC, liver sinusoidal endothelial cell.

Key Figure

Schematic Illustration Depicting Evolution of Physiologically Relevant Models



Trends in Biotechnology

(See figure legend at the bottom of the next page.)

organization, or to allow for a directed structural perturbation to investigate tissue response. In fibrosis modeling, the 3D tissue microenvironment is crucial to recapitulate typical fibrogenic features. The tissue-specific microenvironmental compartmentalization, such as patterns formed by collagen deposition or tissue stiffness, can provide more accurate insight into disease progression. By precisely positioning biological materials and living cells within 3D biofabricated tissue constructs can mimic tissue phenotype and functionality, with high-throughput capability, customized geometries, and tunable mechanical properties.

3D biofabrication technologies commonly used are extrusion [27], light-assisted [digital light processing (DLP) and Two-photon polymerization (TPP)] [28], inkjet (droplet-based) [29], and laser-induced forward transfer (LIFT) bioprinting [30]. Each technique has its pros and cons with respect to cell viability, printing resolution, structural integrity, and materials (mostly biocompatible), resulting in constructs that to a varying extent resemble the mechanical and functional properties of fibrotic tissue. For detailed information regarding the different 3D bioprinting techniques, the reader is referred to previous reviews [31,32].

Using extrusion-based printing, Nguyen and colleagues [33] have developed 3D tissue constructs comprising parenchymal (primary human hepatocytes) and nonparenchymal (HSCs and ECs) cells with long-term functionality and exposure to different fibrogenic-compound-induced liver injury [34]. Although tissue architecture does not recapitulate the native hepatocyte structure of the liver, the pattern of collagen deposition, cytokine production, and fibrosis-related gene expression profiles resemble multiple modes of liver injury. This model therefore replicates partial fibrogenesis mechanisms and could help to identify different drug-induced liver injury pathways. Utilizing this 3D bioprinted model, Organovo has demonstrated its feasibility as a high-throughput model for pharmaceutical drug screening in the preclinical evaluation of certain antifibrotic drugs (<https://organovo.com/wp-content/uploads/2019/01/Organovo-Poster-SOT-Mar2018.pdf>).

Ma and coworkers [35] have developed a novel 3D-hydrogel-based triculture model that combines physiologically relevant cells with a liver-specific 3D microarchitecture. In particular, using DLP-based bioprinting, hiPSC-derived hepatic progenitor cells (hiPSC-HPCs), and endothelial and adipose-derived stem cells are embedded in a 3D hexagonal hydrogel construct with controlled stiffness, resembling the shape of a liver lobule. Cells reorganized and realigned into the hexagonal structure, maintain high viability and enable hiPSC-HPCs maturation (Figure 2).

The biofabrication of the alveoli for development of lung and airway models, on the contrary, remains a technical challenge due to their size and complexity [36]. In this aspect, photodegradable microspheres made of poly(ethylene glycol) (PEG) seeded with alveolar epithelial cells have been developed, which resemble hollow epithelial cysts on the scale of alveoli [37]. Although

Figure 1. Compared to the traditional 2D cell cultures as monolayers on flat surfaces, 3D structures, that is, self-assembled spheroids and organoids form more complex 3D structures reflecting physiological cellular organization and intercellular communication, and can recapitulate physiological developmental processes. Several new technological advancements have been made over past years including biofabrication with tunable 3D cellular organization and micropatterned extracellular matrix allowing for directed structural perturbations; bioreactors/organ-on-a-chips with possibility of superfusion/interstitial perfusion integrated with hydrodynamic, mechanical, and soluble paracrine cues, and advanced imaging technology; vascularized organs with opportunities for controlled perfusion, physiological vascular barrier function and physiological nutrient supply and waste removal to highly physiological cell and matrix organization in organ-derived microtissues including vascularized organoids and precision-cut tissue slices. Comparatively, models with increasing complexity and physiological relevance are not always applicable for high-throughput drug screening but provide in-depth knowledge about disease modeling and are useful for development of personalized treatments and provide insights about the translational potential of the novel drug molecules.

Table 1. Advantages and Disadvantages of Current Classes of Fibrotic Tissue Models

Model	Uses	Advantages	Disadvantages	Refs
2D culture	A. Screening of antifibrotic agent and lineage-mapping studies B. Cell migration and mechanobiology studies C. Coculture studies to investigate paracrine interactions	- High throughput - Ease of handling, multiplexing - Single-cell imaging and analysis - Gene expression profiling - Cell-cell and cell-matrix interaction - Tunable stiffness fabrication	- Lack of complexity - Cell-ECM and cell-cell interaction only in X-Y plane - Physiological irrelevant cell behavior and response due to high matrix stiffness	[7,10] A. Liver [3,4], lung [5], kidney [6] B. Lung [8,9] C. Liver [11], lung [12,13]
3D spheroids	A. Drug screening and toxicity studies B. Mechanistic studies understanding the <i>in vivo</i> and patient mimic fibrotic microenvironment	- Self-assembled 3D cellular organization - 3D cell-cell and cell-matrix interaction - Relatively easy to fabricate - High throughput	- Simplified architecture - Size variation and heterogeneity - Lack of vasculature	[14] A. Liver [15,16] B. Liver [17]
3D organoids	A. Adult stem cells (ASCs) derived organoids: disease modeling. B. iPSC-derived organoids: fibrosis development modeling C. Drug screening and toxicity studies	- Self-assembled 3D cellular organization - Patient-derived (ASCs and hiPSCs): mimic complexity and architecture - Genetical stability - Personalized disease modeling and treatment, and CRISPR genome editing	- Lack of vasculature/perfusion - Limited expansion - Time-consuming: long-term culture - Less amenable to high throughput screening	[18–20] A. Lung [21,22], kidney [25] B. Lung [23], kidney [26] C. Lung [24]
3D bioprinting	A. Fabrication of 3D biomimetic scaffold similar to <i>in vivo</i> situation B. Replication and study of induced fibrogenesis mechanisms at the cellular and physiological level C. 3D cell-cell and cell-ECM interaction studies	- High throughput and long-term cultures - Precision control over size and shape - Tunability of mechanical properties - Cell phenotype, gene expression and functionality preservation - Possibility to combine different types of biomaterials, cells and growth factors	- Lack of vasculature/perfusion - Challenges with cell viability - Challenges with materials for printing: biocompatibility and mechanical properties (structural integrity) - Difficult for high throughput	[27–32,40] A. Liver [33], lung [36,37] B. Liver [34,35], lung [38,39] C. Kidney [41]
Bioreactors and organ-on-chip	A. Studies of induced fibrogenesis mechanisms at the cellular and physiological level under dynamic environment B. Evaluating effects of drugs, due to dynamic tissue microenvironment	- Replicate dynamic <i>in vivo</i> microenvironment including hydrodynamic and mechanical cues - Oxygen and nutrients supply and removal of metabolic waste - Multiplexed with other 3D systems	- Lack of vasculature/perfusion - Difficult to adapt for high throughput	A. Kidney [42,49] A. Kidney [43,44], lung [45–47] B. Kidney [48]
3D vascularized models	A. <i>In vitro</i> study of vascular remodeling and cell migration B. Drug screening	- Chemical transport and cellular diffusion - Enhanced cell/tissue maturation and organization - Selective barrier for drug testing - Multiplexing with microfluidic systems - Possibility to develop patient-specific vascularized model with iPSCs	- Dimensional limitations - Risk of clogging and bursting during perfusion - Limited perfusability - iPSCs maturation in microfluidic devices	[50–52,56–58] A. Kidney [53], liver [55] B. Kidney [54]
Decellularized tissue matrices	A. <i>In vitro</i> culture substrate mimicking native or fibrotic disease microenvironment	- High throughput capacity - Replicate <i>in vivo</i> cell-matrix interaction - Restored native matrix stiffness - Restored native protein, growth factors and structure (depending on the process) - Long-term culture and storage (–80°C) - Dimensional tunability	- Accessibility to the tissue samples - Non-anatomical microenvironment - Does not model cell-cell interaction - ECM loss during procedure - Represent end-stage disease	A. Lung [64,65]
Precision cut tissue slices	A. <i>In vitro</i> culture substrate mimicking native or fibrotic disease microenvironment	- Accurate cell-matrix, cell-cell interactions - Dimensional tunability - High-throughput capacity	- Relatively short-term cultures - Repair and regenerative responses - Lack of intact immune system	[59,60] A. Lung [61], kidney [62], liver [63]

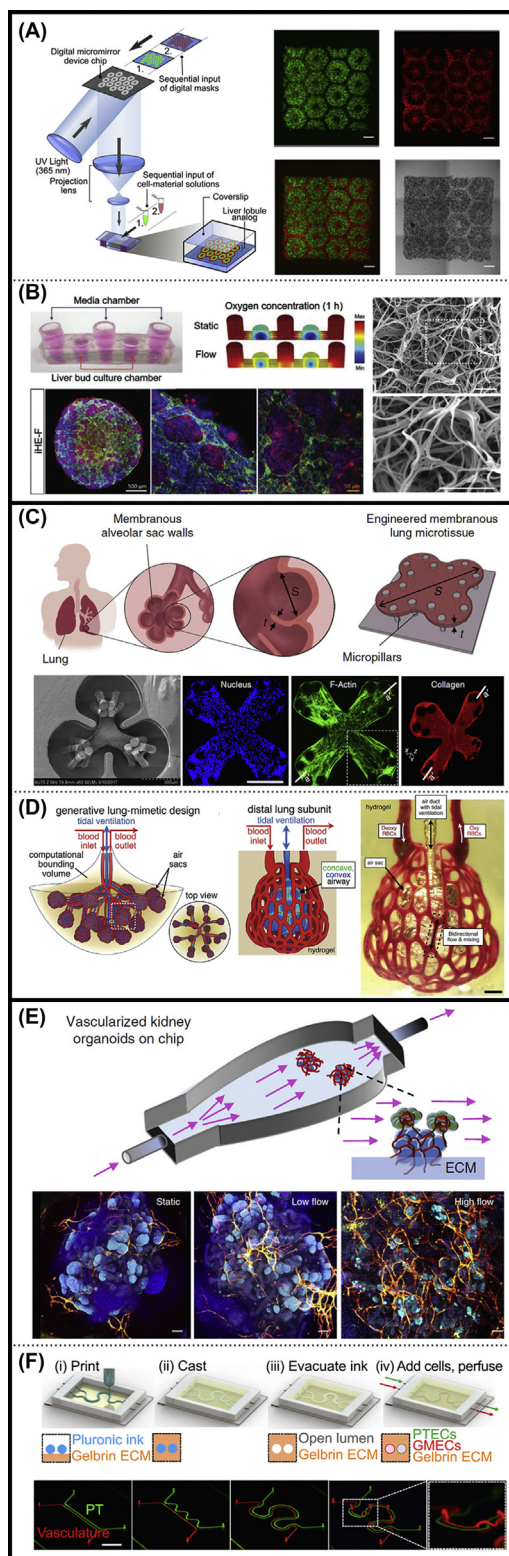


Figure 2. Illustration of 3D Tissue Models for Disease Modeling and Drug Testing. (A) A two-step DMD-based 3D bioprinting of liver lobule structures [38]. (B) 3D vascularized liver organoid composed of iHep cells and a decellularized liver extracellular matrix cultured in a microfluidic system [55]. (C) Engineered lung microtissues that model the membranous morphology of the alveolar sac wall [39]. (D) 3D bioprinted vascularized alveolar lung-mimetic models with multivascular networks and functional intravascular topologies [41]. (E) Bioengineered vascularized kidney organoids in a microfluidic system [56]. (F) Design and fabrication of 3D vascular proximal tubular models [54]. All images reproduced, with permission from [35,38,39,53–55]. Abbreviations: ECM, extracellular matrix; GMECs: glomerular microvascular endothelial cells; PT, proximal tubule; PTEC, proximal tubule epithelial cells.

these constructs insufficiently replicate a hierarchical structure, it has been speculated that these cell-laden microspheres can be combined with 3D-bioprinted alveoli architectural models [38] (Figure 2). In another study, membranous human lung microtissues were developed whereby the key biomechanical events occurring during lung fibrosis, including progressive stiffening, and alveolar tissue contraction accompanied with lung function decline, were recapitulated. In addition, antifibrotic drugs have been evaluated for therapeutic efficacy, suggesting the usability of this model for drug testing [39] (Figure 2).

In order to provide a highly tissue-specific microenvironment, specific-organ-derived decellularized ECM (dECM)-based bio-inks have been developed [40], such as a photo-crosslinkable porcine kidney ECM-derived bio-ink for renal tissue bioprinting [41]. By combining renal dECM with different hydrogel materials, such as gelatin, hyaluronic acid, and glycerol, a printable ink has been developed combining primary human kidney cells, including proximal and distal tubular epithelial cells (PTECs/DTECs) and podocytes. The 3D-bioprinted construct provides structural integrity as well as a tissue-specific microenvironment.

Despite the successful studies, 3D-bioprinted organs have several challenges that must be overcome; for example, bioprinter technology (resolution and speed), biomaterials (biological versus synthetic), cell source (iPSCs versus other cells), and vasculature (for oxygen and nutrient supply).

Microfluidic Bioreactors and Organ-on-a-Chip Technologies

Although organoids or 3D-bioprinted constructs offer great potential, fluid-flow and active mechanical stimulation are not recapitulated in these systems. By combining cells, organoids, or 3D-bioprinted tissues with organ-on-chip technology, (interstitial) fluid flow, mechanical strain, and hydrostatic pressures, which play a central role in fibrosis pathogenesis, can be integrated in the models.

Homan and coworkers created a 3D-perfusible human renal proximal tubule lined with PTECs embedded in ECM in a tissue chip, that showed a long-term maintenance of PTECs phenotype and function, forming a tissue-like epithelium and recapitulating *in vivo* microarchitecture [42]. To mimic the **perisinusoidal space** of the liver, a bioreactor consisting of two plates and a porous membrane was seeded with human umbilical vein endothelial cells (HUVECs) and activated HSCs (LX-2), and subjected to controlled shear stress by applying flow over the HUVECs. This demonstrated that HUVECs stimulated with shear stress inhibit HSCs via paracrine factors [43]. In another approach, Jeong and colleagues constructed self-organizing microhoneycomb structures using natural collagen–Matrigel matrix with hepatocytes and HUVECs spheroids for creating a complex-shape tunable 3D liver model mimicking liver ultrastructure and liver function [44].

Stucki and coworkers developed a breathing lung-on-a-chip model with human alveolar epithelial cells and lung endothelial cells, with passive medium flow and cyclic strain, reproducing unique features of the alveolar microenvironment including breathing motion, air–blood barrier, and air–liquid interface [45]. Mondrinos and colleagues generated a human organ fibrosis chip by encapsulating fibroblasts within collagen hydrogels inside the lung-on-chip device. Chemically induced fibrotic responses were amplified by perfusing monocyte-derived macrophages and cytokines promoting M2 macrophage phenotype [46]. Recently, a 3D-bioengineered pulmonary fibrotic (Eng-PF) tissue was developed utilizing silk collagen-I hydrogels seeded with airway epithelial and microvascular endothelial cells, in combination with a Flexcell bioreactor system [47]. Eng-PF tissue modeled fibroblastic features such as epithelial injury, myofibroblast differentiation, and enabled screening of antifibrotics.

Zhou and coworkers developed a microfluidic and compartmentalized chip device that reproduces the fluidic and 3D microenvironment of the renal proximal tubule [48]. Using immortalized human proximal tubule cells, the epithelial-to-mesenchymal transition involved in the development of renal fibrosis could be recapitulated. Using similar microfluidic device, Kim and colleagues were able to expose kidney epithelial cells to controlled doses of gentamicin and to provide cells with physiological shear stress [49]. It was demonstrated that sublethal doses of gentamicin led to cell injury, causing reduction in tight junctional proteins and increase in the expression of kidney injury marker 1. While this study focused on gentamicin effects, the described method has the potential to be used for a wide range of drug-induced nephrotoxicity studies.

Despite the promising application for drug toxicity studies, many factors remain to be addressed. For example, the limited cell sources and the maintenance of their long-term functionality within the system. Furthermore, a single organ-on-chip model is unable to resemble the main processes involved in pharmacokinetics such as adsorption and drug distribution.

Vascularized Models

An organized and endothelialized vascular network within a bioengineered model is important for providing a physiological microenvironment, but also a physiological selective vascular barrier controlling the molecular transport from the vessels to the surrounding tissue [50]. Furthermore, tissue constructs generated *in vitro* require a constant flow of oxygen and nutrients, and a route to remove wastes, to maintain tissue viability and functionality. However, although the need for a vascular network within a fibrotic tissue model is clear, the engineering and integration of such networks in *in vitro* models remains a challenge.

3D bioprinting represents a valuable tool to address challenges in vascular tissue engineering with the versatility and flexibility to fabricate various designs from a wide range of biomaterials and the precision to control microscale features [51]. Kolesky and colleagues adopted a nozzle-based 3D printer for developing 3D cell-laden vascularized tissues that exceed 1 cm in thickness and can be perfused on a chip for up to 6 weeks [52]. Parenchyma, stroma, and endothelium were integrated within a single thick tissue by coprinting multiple inks composed of human mesenchymal stem cells and neonatal dermal fibroblasts with a customized ECM alongside embedded vasculature lined with HUVECs. In another approach, Zhu and coworkers used a rapid DLP-based bioprinting to create prevascularized tissue constructs with notably high speed, resolution, and scalability [51] for integration within (micro)fluidic devices or bioreactors to simulate blood flow and diffusion.

Recently, Homan and colleagues reported an *in vitro* method for culturing iPSC-derived human kidney organoids under flow on millifluidic chips [53]. They demonstrated that kidney organoids under fluidic shear stress exhibited a highly vascularized tubular and glomerular compartment with maturation of TECs and podocytes. Vascularized kidney organoids emulate key kidney functions, even though the method does not ensure the perfusability of the microvascular networks (Figure 2). In another study, 3D-vascularized proximal tubules were fabricated, using epithelium and endothelium embedded in engineered ECM, that exhibited active reabsorption of solutes via tubular-vascular exchange and vectorial transport of solutes, thereby providing a unique platform for kidney disease modeling and drug testing [54] (Figure 2). Similarly, 3D-vascularized liver organoids composed of iHEP cells, endothelial cells, and hepatic dECM hydrogel cultured in a microfluidic system with a continuous dynamic flow exhibited liver-specific functions [55] (Figure 2).

Different cell types are involved in the formation of a healthy vasculature. Endothelial cells (ECs) form a selective and functional monolayer barrier between the blood and the tissue compartment.

Pericytes and vascular smooth muscle cells play an important role in stabilizing ECs tubes and regulating vascular tone. Where the studies mentioned previously only used ECs for the vascular networks, there were also studies in which all the cellular components were generated from hiPSCs and successfully integrated in microfluidic chips and bioreactors, forming lumenized vessel structures and capillary networks [56,57]. However, the question remains whether hiPSC-derived vascular cells can actually acquire mature characteristics similar to primary cells in engineered microfluidic devices, thus fully replicating the natural barrier function [58].

Physiological Tissue-Based Models

By combining bioprinting using organ-specific bio-inks with organ-specific functional cells and perfused vascular networks, a biomimetic organotypic model can be attained that closely resembles the *in vivo* situation. However, this approach is complex. Therefore, an alternative approach to achieve a physiological tissue model has been developed, referred to as precision-cut tissue slices (PCTSs). PCTS are obtained by cutting fresh tissue into 200–250- μ m thick slices [59]. The major drawback of this system is that the slice preparation results in two-cut surfaces that trigger a repair and regenerative response; for example, HSC activation in liver slices within 96 h [60].

It has been demonstrated that a mixture of profibrotic factors, proinflammatory cytokines, and signaling molecules (TGF- β , **tumor necrosis factor- α** , and platelet-derived growth factor) could induce early fibrosis-like characteristics (except α -SMA induction) in human precision-cut lung slices derived from patients without IPF [61]. Similarly, precision-cut kidney slices (PCKSs) derived from human cortical tissue from tumor nephrectomies [62] demonstrated TGF- β 1-induced renal fibrogenesis characterized by expression of multiple fibrosis markers, including α -SMA. PCKS show maintenance of renal tissue architecture and phenotype (up to 48 h), demonstrating their application as a promising renal fibrosis model.

Paish and coworkers described a novel bioreactor system with extended metabolic activity and functional longevity of precision-cut liver slices, to at least 6 days, without hepatocellular stress and significant spontaneous fibrogenesis [63]. This provides an efficient model for modeling fibrogenesis and testing potential antifibrotic drugs.

In an alternative approach, PCTSs can be decellularized, leaving the organized ECM and, depending on the decellularization method, proteins such as growth factors. Acellular patient-derived scaffolds can retain unique, individual protein composition and structure of their respective disease pathologies (up to 1 month) [64]. Primary human fibroblasts seeded on acellular lung scaffolds derived from IPF patients, displayed myofibroblasts phenotype with increased α -SMA expression. Apart from specific cell–ECM interactions, acellular scaffolds potentially model the extracellular microenvironment of the diseased tissue, including matrix-sequestered growth factors involved in disease onset and pathogenesis [65]. However, scaffolds derived from tissues represent the end-stage disease and are often reseeded with only one or two cell types, thus incompletely modelling cell–cell interactions.

Applications and Future Outlook

Recent advancements have enabled recapitulation of different aspects of human fibrotic diseases, which will significantly contribute to early-stage drug discovery. However, due to the disease complexity, modeling of tissue fibrosis remains challenging, and a clear cut recipe for the ideal model does not yet exist (see Outstanding Questions). Current bioengineered systems often lack the topographical multicellular organization, perfusable (micro)vasculature, multicellular response to injury, and organ-specific immune response, which will need to be integrated into the

Outstanding Questions

What level of complexity is required for *in vitro* fibrosis models? The model should be as simple as possible for high-throughput drug screening, however should be as complex as needed for understanding the disease and to predict the efficacy of drugs in human disease-relevant model.

Although 3D models provide spatial cellular organization, which model is most relevant for *in vitro* fibrosis modeling? Should it be based on self-organization, or imposed cellular arrangement via 3D bioprinting? These questions should be considered during development of the model, even though the answers likely will vary depending on individual applications.

Fibrosis represents different stages from early fibrosis to advanced fibrosis; a critical aspect which is currently not addressed by most developed models. How can fibrosis be staged using these models? This would be crucial to study the drug-induced reversal of fibrosis and liver regeneration.

The integration of a vascular network in a fibrotic tissue model is imperative for oxygen and nutrient supply and as a physiological selective barrier during compound screening. However, can these networks always be included, provided that vascular cells will require different cell culture media supplemented with different growth factors?

Inflammation has been recognized in recent years to play an important role in fibrosis. How can inflammation, which is a systemic process, be implemented within the current bioengineered models that are designed to replicate a specific tissue type?

The future goal of currently developed bioengineered *in vitro* models is personalized disease modelling and development of personalized medicine. How far can we reach this goal with currently developed models? Are fibrotic tissue models based on cryopreserved iPSCs from patients, the future direction to explore?

optimal new generation fibrotic tissue models. However, before designing such complex models, one should define what level of complexity is actually needed in a specific model for a specific application. Even though the addition of complexity, for example, by including a vascular network or immune cells, will make the tissue model more physiological, it is also likely to result in models that are harder to operate, control and evaluate. As such, tissue models should be as complex as needed to answer specific questions, but also as simple as possible to allow for an efficient usage.

Furthermore, the progressive development of robust hiPSC differentiation approaches are promising and will facilitate development of patient-specific fibrotic models for personalized disease modeling and medicines. However, for successful future implementation, strategies for the acquisition of patient-specific iPSCs need to be optimized. Imperatively, integration of bioreactors and micro/millifluidic devices can improve the longevity of bioengineered models and provide high level of control over cellular microenvironment [66]. This control will facilitate the use of guided self-organization for the formation of complex spatially organized tissue models, which is a strong alternative to technologies like 3D-bioprinting where tissue remodeling after fabrication is often difficult to predict and control. Additionally, a temporal control over tissue development could allow for the replication of different stages of fibrosis, resulting in a deeper understanding about disease progression and the possibility to test different fibrosis stage-based treatment options.

Despite the shortcomings, recent advancements in engineered fibrotic tissue and organ models do represent promising platforms for efficiently recapitulating the biological mechanisms involved in fibrosis pathogenesis, providing versatile tools for disease modelling and drug testing in clinically relevant disease models.

Acknowledgments

J.R. received financial support from the European Research Council (ERC) under the European Union's Horizon 2020 Research and Innovation Programme (724469).

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